ドメインライブラリー的発想によるがん治療抗体の開発研究

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氏　　名　杉　山　在　生　人
研究科，専攻の名称　東北大学大学院工学研究科（博士課程）バイオ工学専攻
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論 文 審 査 委 員　主査　東北大学教授　梅津　光央　東北大学教授　中山　亨
東北大学教授　末永　智一

論文内容要約

First section: Introduction

Bispecific antibodies are non-natural antibodies reconstructed from two distinct monoclonal antibodies. The two different Fvs in a bispecific antibody simultaneously bind to two target antigens, and the formation of linkages between the two target antigens on cell surfaces can induce synergistic signals in the cells. For cancer therapy, cross-linking of immune cells with cancer cells induces the immune cells to damage the cancer cells. Especially, bispecific antibodies can target highly cytotoxic T cells, which are not activated by natural antibodies. Because of their abundance, proliferation capacity, and serial killing action, T cells can effectively attack tumors and a bispecific T-cell recruiting antibody can circumvent the mechanisms used by tumors to escape from immune effectors. These advantages of T-cell–recruiting antibodies have driven researchers to generate a large number of these antibodies with different cancer targets and bispecific structure formats; the studies have shown that the cytotoxic activities of these antibodies depend on the antigen target and the antibody structure format; for instance, changing the target can cause a ~10^3-fold difference in cytotoxicity and the cytotoxicity is strongly dependent on the bispecific structure (diabody, single-chain diabody, tandem single-chain Fv, etc) and arrangement of antibody domains. However, the relationships between these factors are complicated, and we have no optimized approach for choosing the appropriate Fvs and domain arrangements to construct bispecific antibodies with sufficiently high cytotoxicity to be clinically effective. In this thesis, we developed a process for screening highly cytotoxic clones by using the T-cell recruiting library, which is comprehensively constructed from domain library; so called ‘domain library approach for generating antibody recruiting T-cell (Dolag ArT) system’.

Second section: Construction of a semi high-throughput process for screening highly cytotoxic T-cell recruiting bispecific antibodies

We constructed a variety of bispecific T-cell recruiting antibodies from a series of the Fvs against T-cell receptors (CD3 and CD28) and the epidermal growth factor receptor (EGFR) family (EGFR, HER2–4), and critical rules of high cytotoxic antibodies are elucidated in the screening process from the clump of bispecific antibodies. We focused on the traditional diabody, which has two single-chain Fv (scFv) fragments with swapped heavy-chain variable (VH) and light-chain variable (VL) domains dimerized to form bispecific antibodies. For each target epitope, we constructed diabodies with the VH and VL domains in different orders, because changing domain arrangement in a diabody can cause more than 10^3-fold cytotoxicity difference. We developed a set of rapid operations for constructing the expression vectors and for expressing and purifying proteins, to make a variety of 100 diabodies with different hetero scFvs and domain arrangements. These prepared diabodies were then screened for high cytotoxicity in MTS assays to ascertain the critical rules for design of antibodies with high cytotoxicity (Fig. 1). The results of this screening process demonstrate that cytotoxicity changes drastically according to the Fv used and the domain order, and provide critical rules for the design of diabodies with high cytotoxicity.

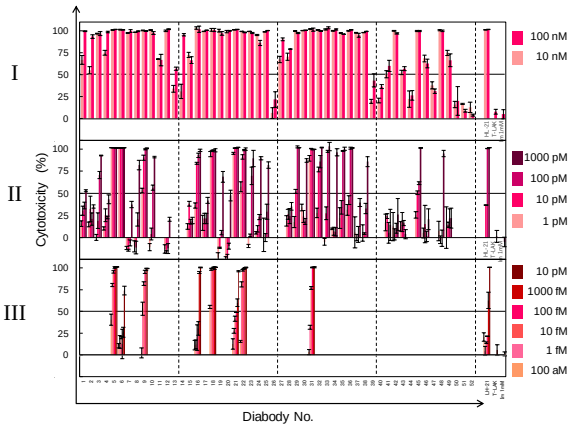

Fig. 1 cytotoxicity screening with IMAC-purified LH-type diabodies; step II was applied for the clones which showed more than 50% cytotoxicity at step I, step III was applied for the clones which showed more than 50% cytotoxicity at step II,

Third section: Dolag ArT systems for high throughput screening of cytotoxicity and binding affinity without purification process

We improved the high-throughput performance of this screening process by removing protein purification process and adding a stage for determining the diabody concentrations in culture supernatant. The diabodies prepared in the second section contained tandemly arranged peptide tags at the C-terminus, which allowed the concentration of diabodies in the culture supernatant to be detected by using a tag-sandwich enzyme-linked immunosorbent assay. When using a tag-sandwich ELISA, careful selection of the capture and detection antibodies was needed. Here, we found that the sensitivity of the assay was affected by the Fvs and the structural configuration of the diabodies; however, the diabody concentrations estimated

from the tag-sandwich ELISA was sufficiently accurate for screening the cytotoxicity of unpurified diabodies in our high throughput method (Fig. 2).

Surface plasmon resonance measurement allowed relative binding affinity to be determined for unpurified diabodies in culture supernatant, and the relative binding affinities of the unpurified diabodies were comparable to those of the purified antibodies. Thus, in this section, we improved the high-throughput performance of cytotoxicity screening and added the analysis process of the binding parameters of highly cytotoxic bispecific antibodies in the screening method constructed in the second section, which we call Dolag ArT (**Do**main **l**ibrary **a**pproach for **g**enerating **A**ntibody **r**ecruiting **T**-cell).

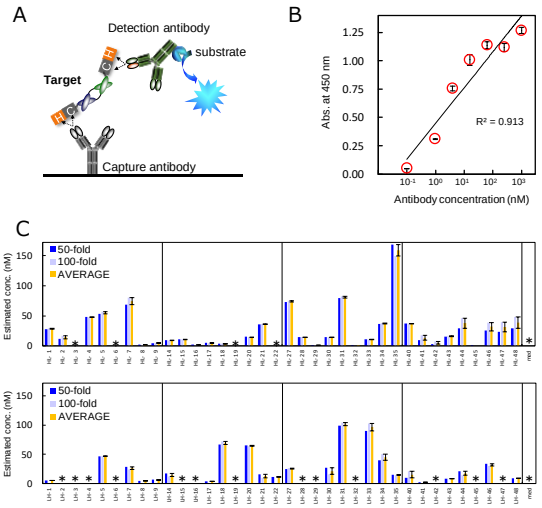

Fig. 2 (A) Schematic representation of the tag-sandwich enzyme-linked immunosorbent assay (ELISA), (B) Detection of diabody HL-31 by means of tag-sandwich ELISA. (C) Estimation of diabody concentration in culture supernatant estimated from 100- and 50-fold-diluted supernatants. Undetectable diabodies are indicated with asterisks.

Fourth section: Construction of T-cell reporter assay with the potential of combining in Dolag ArT system

We constructed a cell reporter assay for measuring T-cell activation induced by diabody format which could be applied in the Dolag ArT system. The activation signals in T-cell were only detected by adding diabodies in the coexistence of T-cells and cancer cells. When this T-cell reporter assay for 72 diabodies used in the third section was applied, the expressed amount of 42 diabodies were enough for detecting the T-cell activation signal, implying the potential for incorporating the Dolag ArT system. By applying the T-cell reporter assay for 9 diabodies with various properties of binding affinity to EGFR, strong correlation between binding affinity to EGFR and T-cell activation ability was observed. On the other hand, there was no correlation between binding affinity to CD3 and T-cell activation ability. These results indicate that T-cell activation ability depends on the binding affinity of diabody for cancer cell. In addition, we reveled that T-cell activation ability is one of the major factors to determine the cytotoxicity against cancer cells. Therefore, the importance of selecting the domain with high binding ability for cancer was demonstrated when T-cell recruiting antibodies are designed for cancer therapy.

Fifth section: Combination of Dolag ArT and library approach

Dolag ArT system has demonstrated the effectiveness for screening promising clones from the library of T-cell recruiting antibody. Here, we combined Dolag ArT and library approach: we generated the domain library of cancer-binding domains by using phage display method, and the positive library applied for Dolag ArT. Firstly, we tried to get the antibody fragment with affinity for cancer target by means of M13 phage display technology. Positive phage clones were amplified via biopanning, and the gene fragments of positive antibodies were simultaneously ligated into the gene fragments of bispecific antibodies with anti-CD3 antibody fragments to make polyclonal bispecific antibody genes. This process enabled to construct around 300 positive bispecific antibodies and to isolate bispecific antibodies directly by screening cytotoxicity. Consequently, we got several high cytotoxic BiBian clones, and we demonstrated developed process is efficient for screening promising clones.

Sixth section: Conclusions

We constructed 'Dolag ArT' system for screening promising T-cell recruiting antibodies focused on cytotoxicity, binding affinity and T-cell activation ability. This system enabled us to screen several promising clones for T-cell recruiting antibodies with high throughput and probability. In the future, various T-cell recruiting antibodies would be developed by means of Dolag ArT system.